The Effects on the Fetus of Diphenylhydantoin Injected During Preimplantation Into Mice

An Honors Thesis (HONRS 499)

by

Kelley L. Wolter

Dr. Clare Chatot

Clare L. Chatot

Ball State University

Muncie, Indiana

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Abstract

Epilepsy is a chronic nervous disorder caused by abnormal electrical signals in the brain. Epileptic seizures can be minimized with the use of anticonvulsant drugs. Use of anticonvulsants by a pregnant woman poses the risk of damage to an unborn baby. One of the most common anticonvulsants is diphenylhydantoin (DPH).

To study the effects on the fetus of preimplantation exposure to anticonvulsants, DPH was injected into A/J and NSA strains of mice during the first five days of pregnancy. Fetuses were examined externally on Day 18 of development and later dissected for internal examination. The DPH mice of both strains showed no internal or external anomalies other than reduced size. Other effects found were the inability to establish pregnancy, increases in resorptions, and smaller litter size.

Introduction

In order to understand the effects on the fetus of diphenylhydantoin injected during preimplantation into mice, it is necessary to understand background information. Therefore, information will first be given on epilepsy, the risks of anticonvulsant use, factors involved in expression of anomalies, and the teratogenicity of diphenylhydantoin.

Epilepsy

Epilepsy is a chronic nervous disorder associated with abnormal electrical activity in the brain (Encyclopedia Americana 1993). The known causes of epilepsy are inherited diseases, inherited tendency for epilepsy, fetal exposure to drugs, head injuries, brain tumors, and blood clots in the brain (Gumnit 1990). According to Gumnit (1990), about 2% of the general population develops epilepsy by age 40. According to another source (Finnell 1981), 0.3-0.5% of the population consists of epileptic women. There are two types of epilepsy, namely, generalized and partial (Sands and Minters 1977). Generalized epilepsy is when seizures involve the whole brain. Partial epilepsy is when seizures involve or begin in one area of the brain. In a French study, researchers classified 6,000 private epilepsy patients. One quarter could not be classified but of the remaining

patients, 37.7% had generalized epilepsy and 62.3% had partial epilepsy (Gastaut et al. 1975). Epileptic seizures can be minimized with the use of anticonvulsant drugs.

Risks of Anticonvulsant Drug Use

There are over 20 anticonvulsant drugs, including; diphenylhydantoin (DPH or more commonly Dilantin or phenytoin), carbamazepine, phenobarbital, valproic acid, trimethadione (Tridione), primidone, phensuximide (Milontin), among others. DPH, valproic acid, Tridione, carbamazepine, and combinations of these pose the greatest risk for congenital malformations in humans (Finnell et al. 1992). Congenital malformations are a result of the ability of anticonvulsant drugs to pass through the placenta to the embryo (Speidel and Meadow 1972, Melchior et al. 1967, Mirkin 1971). Use of anticonvulsants increases the frequency of occurrence of congenital malformations two to three-fold (Speidel and Meadow 1972, Lowe 1973). One in 400 women use anticonvulsants and 2/3 of women that use anticonvulsants, use DPH either alone or in combination with other anticonvulsants (Biale et al. 1975). DPH has been shown to cause congenital malformations in mice (Massey 1966, Gibson and Becker 1968, Harbison and Becker 1969). Finnell (1981) showed that

Fetal Hydantoin Syndrome is caused by the drug and not the disorder with an "epileptic" mouse model. Mice not treated with DPH that had regular convulsions during pregnancy gave rise to normal offspring. In humans, up to 30% of epileptic mothers on anticonvulsant medication give birth to babies with fetal hydantoin syndrome (Hanson 1986). The most common congenital malformations associated with the fetal hydantoin syndrome are: craniofacial anomalies, prenatal and postnatal growth deficiencies, mental retardation, and limb defects (Buehler et al. 1990, Hanson 1982). Other anomalies that occur with less frequency include; microcephaly, ocular defects, cardiovascular anomalies, hypospadias, and umbilical and inguinal hernias (Buehler et al. 1990, Hanson 1986, Jones 1988). Although there is a risk of fetal malformation associated with the use of anticonvulsant drugs, the damage to the fetus from anoxia produced by a seizure could be greater (Lowe 1973, Monson et al. 1973).

Expression of Anomalies and the Teratogenicity of Phenytoin

Expression of anomalies depends on the period during gestation when exposure to anticonvulsants occurs, dose, duration of exposure, and genetic predisposition (Wilson 1977, Finnell and Chernoff 1984). Harbison and Becker (1969) showed that single administration of DPH to pregnant

Swiss-Webster mice on days 9-14 of gestation gave rise to various fetal anomalies, growth retardation, and sometimes death. Finnell (1991) suggested that structural defects could only be induced during limited "windows of sensitivity" which change over gestation for the different organ systems.

Expression of anomalies is affected by the dosage of anticonvulsants taken (Wilson 1977, Finnell and Chernoff 1984). In a study by Hill et al. (1974) mothers who used the combination of anticonvulsants, primidone and DPH, had the greatest number of life-threatening or disfiguring anomalies (three of six infants). In this same study, only two of nine infants treated with DPH alone had serious anomalies. Lowe (1973) and Fedrick (1973) also observed that a combination of anticonvulsants increased the risk of producing a child with defects. Additionally, Fedrick (1973) noted a dosage response with phenobarbitone but not with phenytoin suggesting that the parent drug may not be the proximal teratogen.

The duration of treatment before conception also may play a role in the expression of anomalies. Hill et al. (1974) observed that the infants most

severely affected were born to mothers who had a history of seizures for an average of 21 years.

Recently, more emphasis has been placed on the role of genetic predisposition for expression of anomalies. Individual fetuses with an enhanced sensitivity to a teratogen-induced pattern of malformation may have mutations in a specific biochemical pathway that alters the metabolism with a subsequent deleterious effect on embryonic development (Finnell 1991). The teratogenicity of phenytoin is believed to be mediated by the toxic arene oxide intermediary metabolite, not by the parent compound (Buehler et al. 1990, Blake and Martz 1980, Martz and Fallinger 1977, Wells and Harbison 1980). The biotransformation of phenytoin to its toxic oxidative metabolites is regulated by a cytochrome P450 enzyme system (Finnell 1992). Toxic arene oxide metabolites are highly reactive and can covalently bind to DNA, RNA, and protein to cause a disruption in normal development (Jerina and Daly 1974, Martz et al. 1977, Oesch 1976, Shanks et al. 1989, Spielberg et al. 1981). Fetuses, when stressed in utero by phenytoin, do not readily metabolize the arene oxide metabolite making them substantially more susceptible to the teratogenic effects of this compound (Buehler et al. 1990). By measuring the epoxide

hydrolase activity with chromatographic assay in randomly selected amniocytes from 100 pregnant women, Buehler et al. (1990) found a trimodal distribution of enzyme activity (Figure 1). This suggested three genotypes which is found in a one gene, two allele system. Buehler et al. (1990) found that amniocytes with low epoxide hydrolase activity (homozygous recessive) were the most susceptible to fetal hydantoin syndrome. Heterozygotes were better able to metabolize the drug and the homozygous dominants were at minimal risk for congenital malformations.

Objectives of this Study

This experiment will attempt to show the effects of phenytoin on the fetus when injected preimplantation. Morphological differences, litter size, and percentage of no litter in DPH versus Control for the NSA Harlan strain and the A/J strain will be noted. Additionally, morphological differences will be noted for females versus males.

Methods and Materials

Mating the Mice

Two females (NSA, Harlan Sprague Dawley, Indianapolis, IN or A/J, Jackson Lab, Bar Harbor, ME between the ages of 6.5-22 weeks) per one

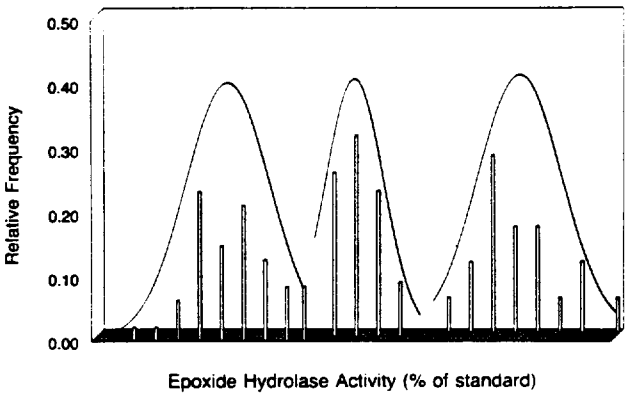


Figure 1. Trimodal distribution of epoxide hydrolase activity in the normal population (Buehler et al. 1990).

male (B6SJLF1/J, Jackson Lab, Bar Harbor, ME) were placed in a cage in the afternoon. They were allowed to mate overnight and in the morning the females were checked for sperm plugs. Plug positive females were separated from plug negative females. Half of the plug positive females were used as controls and half as experimentals.

Injections

The controls were injected intraperitoneally with .01 N NaOH. The experimentals were injected with 50 mg/kg of phenytoin dissolved in .01 N NaOH. Injections were given for five days beginning on the day they were found to be plug positive (day 0). Injections were given at the same time each day ± one hour.

Dissection of Fetuses from Mother

On Day 18 of gestation, the mice were anesthetized with chloroform and were sacrificed by cervical dislocation to prepare for fetal dissection. The abdomen was cleansed with 70% alcohol and the skin was cut away to expose the viscera. The uterine horns were externalized and examined. The mother's ovaries were checked for the number of corpora lutea and the uterine horns were checked for implantation sites and for any resorptions. The number of fetuses per side of the uterus and total

number of fetuses was noted for each mouse. The fetuses were removed individually and placed in a dish with 0.9% sterile saline.

Initial Analysis of Fetuses

Fetuses were checked for any gross morphological defects and then were weighed in grams. Crown to rump length was measured in millimeters. Litters were stored together in Carnoy's fixative for at least a week before proceeding to the next step.

External Morphological Analysis

Before dissection of the fetus, several external features were noted. Right arm and right leg lengths were measured with a metric ruler from insertion into the body to the proximal end of the phalanges. The fourth phalange distally on the right front and right hind leg was measured to the nearest 0.5 mm. The distance between the eyes across the bridge of the nose was measured along with the crown to rump length. Also, the mouth was checked for cleft lip.

Dissection of the Fetus

1. Head

The first cut on the fetus was made with a scalpel that separated the upper and lower jaws and slanted slightly to the neck. The tongue was

then removed and the palate was examined for a cleft. Then, one millimeter transverse sections were made through the head beginning at the nasal region. The first transverse section revealed bilateral nasal cavities that were checked for the normal open, vertical and uniform shape (the vomer being perpendicular to the palate). Another cut was made that passed through both eyes. Eyeballs were checked for uniformity and round shape. Another section was made between the frontal and parietal bones that revealed the lateral and third ventricles. The ventricles were checked for abnormal enlargement (internal hydrocephalus) or shrinkage (external hydrocephalus). The final cut was made to expose the cerebellum which was examined for gross morphological defects. Some other cuts were made that fell between these important sections in order to maintain the one millimeter thickness of the sections.

2. Body

Beginning in the neck region, the first transverse section was made to skip most of the glandular area. The next section was made to go through the heart. The interventricular septum was checked with a dissecting microscope for defects and the direction of the aortic arch was checked.

The lungs, liver, stomach, pancreas, kidneys, intestines, and bladder were all checked for gross morphological defects. The reproductive system was examined and the sex was determined.

Skeletal Analysis

Fetuses from each litter were used for this analysis after being fixed. They were eviscerated and skinned and were placed in a series of solutions to disintegrate visceral tissue and dye skeletal portions. The order is given here:

1. 2% KOH (3.5 days)
2. Stain* (7 days)
3. 37°C incubator in stain (8 hours)
4. 2% KOH/25% glycerol (2 days)
5. 2% KOH/50% glycerol (2 days)
6. 50% glycerol (2 days)
7. 100% glycerol (storage)

*The recipe for stain (Kimmell 1981) with a doubling of the Alcian blue and Alizarin red for this experiment is as follows:

4 parts Alcian blue 0.14% in 70% ethanol, filtered (stains cartilage)
2 parts Alizarin red 0.12% in 95% ethanol, filtered (stains bone)
8 parts Glacial acetic acid
50 parts Ethanol

Results

For the first NSA group, female mice (22 g) were mated at age 9.5 weeks. Six mice were plug positive so three were used as experimental

and three for control. The experimentals were injected with 75 mg/kg (0.1 ml) of DPH and all three died. The controls were injected with 0.1 ml of 0.01 N NaOH. The first control mouse yielded 13 fetuses, the second had 11 fetuses, and the third had zero fetuses and zero resorptions. This dose of DPH was toxic to adults therefore the dose was reduced to 50 mg/kg.

For the second NSA group, female mice (22 g) were mated at age 10.5 weeks. Two mice were plug positive and both were used as experimentals. The experimentals were injected with 50 mg/kg (0.06 ml) of DPH. The first mouse yielded five live fetuses and two resorptions. The second mouse had zero fetuses.

For the first A/J group, female mice (16 g) were mated at age 6.5 weeks. Six mice were plug positive so three were used as experimentals and three for controls. The experimentals were injected with 55 mg/kg (0.04 ml) of DPH. The first DPH mouse yielded three fetuses, the second and third both had zero fetuses and zero resorptions. The controls were injected with 0.04 ml of 0.01 N NaOH. The first and third mice had zero fetuses and zero resorptions. The second mouse had one fetus and one resorption.

For the second A/J group, female mice (16 g) were mated at age 7.5 weeks. Eight mice were plug positive so four were used as experimentals and four for controls. The experimentals were injected with 55 mg/kg (0.04 ml) of DPH and three of them died. The fourth mouse yielded five fetuses. The controls were injected with 0.04 ml of 0.01 N NaOH. The first control mouse yielded eleven fetuses with one of them being a runt. The second control yielded zero fetuses and seven resorptions. The third mouse had eight fetuses and the fourth had twelve fetuses.

For the third A/J group, female mice (16 g) were mated at age 15 weeks. Two mice were plug positive and both were used as experimentals. They were injected with 55 mg/kg (0.04 ml) of DPH. The first mouse yielded ten fetuses, one of which was not fully formed and dead. The second mouse had zero fetuses and seven resorptions.

For the fourth A/J group, female mice (16 g) were mated at age 22 weeks. Two mice were plug positive and both were used as experimentals. They were injected with 55 mg/kg (0.04 ml) of DPH. The first mouse yielded zero fetuses as did the second but the second had three resorptions. Table 1 summarizes this data.

	NSA Group 1	NSA Group 2	A/J Group 1	A/J Group 2	A/J Group 3	A/J Group 4
wt. of mother	22 g	22 g	16 g	16 g	16 g	16 g
mating age	9.5 weeks	10.5 weeks	6.5 weeks	7.5 weeks	15 weeks	22 weeks
# plug positive	6	2	6	8	2	2
DPH injection	75 mg/kg	50 mg/kg	55 mg/kg	55 mg/kg	55 mg/kg	55 mg/kg
Control inject.	0.1 ml		0.04 ml	0.04 ml		
# in DPH litters		5(2), 0	3, 0, 0	5	9(1), 0(7)	0, 0(3)
# in Cont. litter	13, 11, 0		0, 0, 1(1)	11,0(7),8,12		
Note	DPH dose too			3 DPH mothers		
	high. All 3			died.		
	mothers died.					

Table 1. Summary of the mating and litter data. Numbers in parentheses represent the number of resorptions in that litter. DPH dosage was reduced because of maternal death and to account for the smaller size of the A/J strain.

	weight (g)	CR length (mm)	eye dist. (mm)	arm (mm)	leg (mm)
NSA DPH (n=5)	0.565	16.6	3.7	3.9	4.2
NSA CONT. (n=24)	1.138	21.8	4.1	4.8	5.6
A/J DPH (n=18)	0.864	19.8	4.1	4	4.3
A/J CONT. (n=32)	0.969	20.8	4.2	4	4.8

Table 2. Averages of morphological data. DPH exposure caused a decrease in both strains of weight, CR length, and eye distance. Additionally, in the NSA strain arm and leg lengths were reduced.

Morphological data (weight, crown to rump length, eye distance, arm length, leg length, finger length, toe length, and sex) were collected and the averages are shown in Table 2. Control vs. DPH in the NSA strain had significantly different results ($p \leq .05$) using an independent t-test. DPH treated fetuses showed significant growth retardation (Figure 2) relative to controls. Weight averages, CR length averages, and leg length averages showed highly significant differences ($p=2.3 \times 10^{-12}$; $p=3.2 \times 10^{-8}$; p=.0007, respectively) compared to controls. Eye distance and arm length were also significantly reduced (p=.014; p=.016, respectively). Control vs. DPH in the A/J strain showed less of an overall growth retardation and only a slightly significant difference in CR length (p=.050) relative to A/J Controls.

There were reductions in the litter size of the DPH treated animals in both strains (Table 3). NSA Control had an average litter size of 12 and NSA DPH had an average of 5. A/J Control had an average litter size of 8 and A/J DPH had an average of 6. There was not a significant difference in the litter size of A/J compared to NSA. A comparison between strains and between females and males of DPH vs. Control yielded no significant differences.

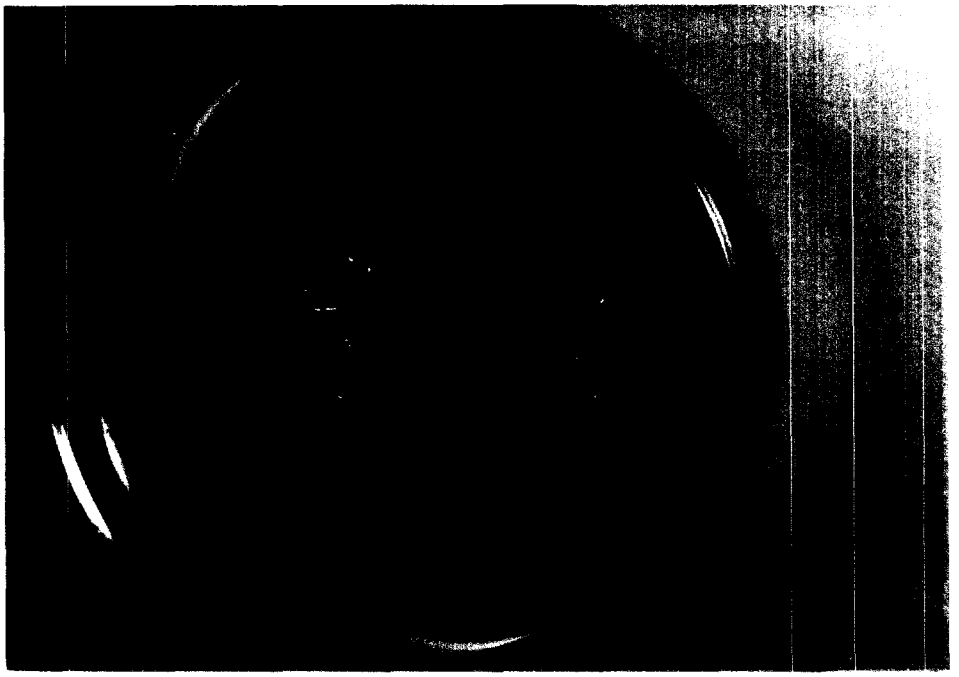


Figure 2. Overall growth retardation in the NSA DPH (left) as compared to the NSA Control (right).

CONTROL	litter size		DPH	litter size
NSA #1	13		NSA #1	5
NSA #2	11			
average	12		average	5
A/J #1	11		A/J #1	3
A/J #2	1		A/J #2	5
A/J #3	12		A/J #3	(10)
A/J #4	8			
average	8		average	(6) 4

Table 3. Litter size and average litter size of DPH vs. Control.
The average in parentheses is if A/J #3 is included in the calculation.

	% no litter	% resorptions	
NSA Control	33% (1/3)	0% (0/24)	
NSA DPH	50% (1/2)	28% (2/7)	
A/J Control	42% (3/7)	20% (8/40)	
A/J DPH	62.5% (5/8)	38% (11/29)	

Table 4. Percent no litter and percent of resorptions.
Both strains show an increase in % no litter and
% resorptions with exposure to DPH.

Percentages of no litters and number of resorptions are shown in Table 4. DPH in both strains showed a higher percentage of no litter and a higher number of resorptions. NSA had 33% no litter and 0% resorptions in the control group. NSA with DPH treatment increased the percentages to 50% no litter and 28% resorptions. The A/J Control had 42% no litter and 20% resorptions. The A/J DPH increased to 62.5% no litter and 38% resorptions.

Sectioning of the fetuses revealed no internal anomalies. It did, however, reinforce the previous measurements showing overall growth retardation by the reduced size of internal features (Figures 3 and 4).

Discussion

Most research on the effects of DPH focuses on the postimplantation stages of development when the embryo undergoes rapid growth. This experiment deals with the effects of DPH during preimplantation. Preimplantation in the mouse has seven stages: 1-cell, 2-cell, 4-cell, morula, early blastocyst, and late blastocyst. Implantation is the embedding of the developing blastocyst into the uterine mucosa and it occurs 4.5 days after fertilization (Taylor 1986). Because the

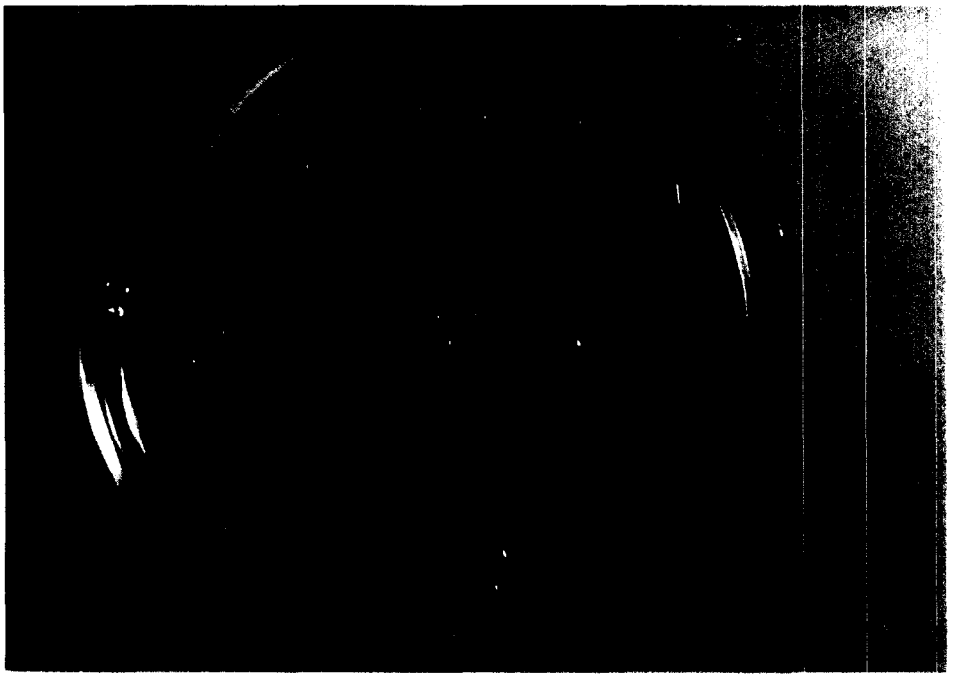


Figure 3. Transverse sections of an NSA DPH.

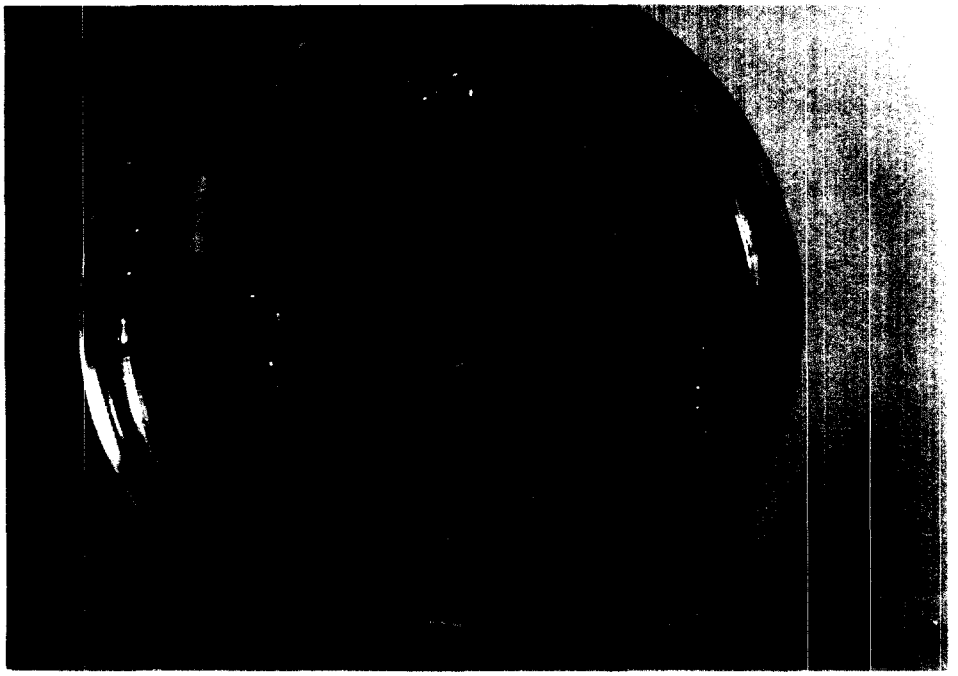
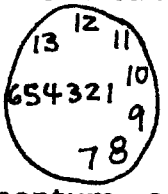


Figure 4. Transverse sections of an NSA Control. Important aspects of the sections in Figures 3 and 4 can be found using this diagram:



1-2 nasal cavities, vomer, palate
3 eyes
4-5 lateral and 3rd ventricles
6 cerebellum
8-9 interventricular septum, aortic arch,lungs
10-11 liver, stomach, pancreas, kidney
12-13 intestines, bladder, reproductive system

blastomeres of the developing embryo are equipotent up to the early 8-cell stage, it has been postulated that the effects of DPH during preimplantation are "all-or-none". This theory is not well supported in this case because of the wide range of expression of anomalies associated with the use of DPH.

Differences in strains of mice also contributes to the range of anomalies expressed. In this experiment, two strains of female mice were used, A/J and NSA. The A/J strain is an inbred strain that is prone to phenytoin-induced cleft palate anomalies (Finnell 1991). The NSA strain is a randomly bred strain with varying sensitivities. The NSA strain in this experiment was very sensitive and had a large response to the treatment. The A/J strain did not show any cleft palate anomalies and, in general, did not respond as well to treatment as the NSA strain.

The NSA morphological differences between Control and DPH were highly significant. The weight, CR length, eye distance, arm and leg lengths were all substanstially smaller in the DPH treated group. No other anomalies were noted. Finger and toe lengths were measured but because of their small size relative to the measuring instrument, no significant difference was noted.

The A/J morphological differences between Control and DPH were not as pronounced. CR length was the only measurement that was significant and its significance was only slight. One A/J litter was abnormally large in size compared to the others. If this data is omitted, there is a significant difference in leg length. The unusually large size may be attributed to the mother's ability to metabolize the drug. If this is true then the mother may have been a heterozygote for arene oxide metabolizing activity allowing less of the drug to cross the placenta to affect the development of the fetuses.

Epileptic women have an increased rate of spontaneous abortions (Nakane et al. 1980) that is amplified by the use of anticonvulsant drugs. The mice in this experiment showed early and late disruption of development as shown by the percent no litter and the number of resorptions. The DPH group in both strains showed a greater amount of no litter and resorptions than did the Control group. The significant difference in litter size is a reflection of these disruptions in development of the fetuses. The difference between strains was not significant.

Skeletal analysis of the Control group showed normal development but due to time constraints no examination of the DPH group was made. Other studies using postimplantation dosing have found skeletal anomalies such as inhibition of skeletal growth, lack of fusion of sternabrae, and fused vertebrae (Harbison and Becker 1969, Dabee et al. 1975).

From this study, it has been shown that diphenylhydantoin does have an effect on the fetus when injected during preimplantation. Effects shown are the inability to establish pregnancy, increases in resorptions, small litter sizes, and overall growth retardation. These findings are supported by observations in other studies and, in humans, reinforces the need for epileptic women on anticonvulsants to consult a doctor in preparation for pregnancy.

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